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Extracytoplasmic adenylate cyclase of *Bordetella pertussis*

(exoenzyme/malate dehydrogenase/alkaline phosphatase/bacterial enzyme compartments/adenosine 3′:5′-cyclic monophosphate)

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ABSTRACT **Soluble adenylate cyclase [EC 4.6.1.1] accumulates in the culture medium of exponentially growing *Bordetella pertussis* (300–900 pmol of cAMP formed/min per ml of 24 hr culture supernatant). In addition, there is an extracytoplasmic adenylate cyclase which enables the intact organisms to form [^{32}P]cAMP (adenosine 3′:5′-cyclic monophosphate) from exogenous [α-^{32}P]ATP (200–1200 nmol of cAMP formed/min per g wet weight of cells) and which comprises 20–45% of the total adenylate cyclase activity. In contrast, only 1.7 and 2.4% of the total cell malate dehydrogenase [EC 1.1.1.37] and alkaline phosphatase [EC 3.1.3.1], respectively, are detectable in the intact cell. Trypsin treatment of intact organisms destroys 96% of the extracytoplasmic adenylate cyclase, but does not reduce the total cell malate dehydrogenase or a small pool of intracellular adenylate cyclase. Four compartments of adenylate cyclase in *B. pertussis* are proposed; (*A*) soluble enzyme in the culture supernatant (up to 20% of the total activity); (*B*) enzyme associated with intact cells and measurable without cell disruption (20–45%); (*C*) extracytoplasmic enzyme sensitive to trypsin, but not measurable in intact cells at standard substrate concentrations (40–60%); and (*D*) intracellular enzyme (7–9%). In comparison with previously studied bacterial adenylate cyclases, the extracytoplasmic location appears to be unique to the *B. pertussis* enzyme.**

A soluble, adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] has recently been purified from the culture supernatant of exponentially growing *Bordetella pertussis* (1). The enzyme had a molecular weight of 70,000, was inhibited by fluoride, and unaffected by pyruvate or other α-keto acids. Its presence in the culture medium suggested that it might be an exoenzyme, in contrast to previously studied bacterial adenylate cyclases which have been shown to be either soluble (*Brevibacterium liquefaciens* and *Streptococcus salivarius*) or membrane-bound (*Escherichia coli*) intracellular enzymes (2–5).

A number of other bacterial enzymes have been shown to be extracytoplasmic† in location (6). Of these, alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] is among the best studied and has become a prototype bacterial exoenzyme, being measurable in some intact bacteria with the use of substrates known to be excluded from entry into the cell (7, 8). Expression of the enzyme activity by intact cells, in combination with the release of these alkaline phosphatases into the culture medium during growth or with nonlethal perturbations of the cell envelope, has been used to support the idea that these enzymes are located external to the cytoplasmic membrane (7–11).

The present study shows that, in addition to the culture supernatant enzyme and a small amount of intracellular enzyme, a large component of the *B. pertussis* adenylate cyclase can be measured by exposure of the intact cells to [α-^{32}P]ATP and can be destroyed by brief trypsin treatment of the intact cells. Since it has been shown previously that exogenously supplied intact nucleoside triphosphates are excluded from bacterial cells (12), it is proposed that the adenylate cyclase activity expressed by these intact cells represents enzyme in a location external to the cytoplasmic membrane.

Abbreviation: cAMP, adenosine 3′:5′-cyclic monophosphate.

† Extracytoplasmic refers to all enzyme external to the cytoplasmic membrane, including the extracellular; extracellular refers to the enzyme in the medium not associated with the cell.

MATERIALS AND METHODS

Materials. Radioisotopes used were [α-^{32}P]ATP (10 Ci/mmol) from ICN and [^{3}H]cAMP (adenosine 3′:5′-cyclic monophosphate) (0.044 Ci/mmol) from New England Nuclear. Phosphocreatine, creatine phosphokinase (crystalline) and purified *E. coli* alkaline phosphatase were purchased from Sigma. Porcine heart malate dehydrogenase (L-malate:NAD† oxidoreductase, EC 1.1.1.37), *N*-[Tris(hydroxymethyl)-methyl]glycine (Tricine), *p*-nitrophenyl phosphate and reduced β-NAD were obtained from Calbiochem. Salt-free lysozyme (11,000 units/mg), deoxyribonuclease I (1400 units/mg), crystalline bovine pancreas trypsin (180–220 units/mg), and lima bean trypsin inhibitor were purchased from Worthington.

Growth of the Organisms. *B. pertussis* (strain 114) was grown on a rotating shaker for 20–24 hr at 35.5° in Stainer-Scholte medium (13), modified to contain 1.525 g/liter of Tris base. *E. coli* (Crooke's strain ATCC 8739) and *B. liquefaciens* (ATCC 14929) were grown by stationary and shaking culture at 35.5° in Bacto-Synthetic AOAC Broth (Difco) with 0.1% glucose, Bacto-Tryptic Soy Broth (Difco) with 0.1% glucose, and modified Stainer-Scholte medium with and without 0.1% glucose. Bacteria were harvested by centrifugation at 6000 × *g* for 30 min at 4°, resuspended to 2–3 mg of wet weight per ml, and the supernatant medium and resuspended cells were used immediately.

Treatment of the Organisms. Spheroplasts were prepared from fresh cells by treatment with EDTA and lysozyme, and disrupted by osmotic shock.‡ The resulting spheroplast lysate was treated with DNAase‡ and assayed immediately. For other experiments, the cells were disrupted by sonification (Sonifier Cell Disruptor, model W140D) in 20 sec bursts. The adenylate cyclase activity reached a maximum at 80–120 sec, whereas the malate dehydrogenase activity was not maximal until 120–160 sec of sonication. Therefore, the organisms were sonified a total of 160–200 sec. All cell breakage experiments with *B. pertussis* were carried out at least three times. Toluene treatment of intact organisms was carried out by the method of Harwood and Peterkofsky (14). Cells were harvested by centrifugation or filtration (0.45 μm Millipore filter) and resuspended in 60 mM Tricine buffer containing 20 mM K_2HPO_4 (final pH 7.4). Samples were removed for adenylate cyclase assay before and after incubation for 5 min with 1% toluene on an orbital shaker

‡ C. R. Manclark, M. A. Urban, and E. P. Summers, manuscript in preparation.

at 25°. For trypsinization, the intact *B. pertussis* organisms were incubated with 20 μg/ml of trypsin for 5 min at 37°, and the reaction was stopped with 20 μg/ml of lima bean trypsin inhibitor.

Adenylate Cyclase Assay. Adenylate cyclase activity was measured by the conversion of [α-^{32}P]ATP to [^{32}P]cAMP. The method described previously (1) was modified only by addition of a 2 min incubation of the organisms and buffer prior to the initiation of the reaction with mix containing labeled and unlabeled ATP, Mg^{2+}, and an ATP regenerating system. The reaction, with each sample in triplicate, was carried out at 30° and continued for 10 min. The cAMP production increased linearly with an increasing concentration of whole cell protein up to three times the standard assay concentration. The cAMP formed was isolated by the double column method of Salomon *et al.* (15). In a separate experiment, the reaction product was demonstrated to be cAMP by paper chromatography as described by Ide (16).

Marker Enzyme Assays. Malate dehydrogenase was assayed by the method of Ochoa (17). The reaction was begun with 20 μl of cells or sonified cells and the decrease in absorbance was followed from 20 to 80 sec at 340 nm. Results are expressed in μmol of NADH oxidized/min per g wet weight of cells. At maximum malate dehydrogenase activity in cell lysates, 20% of the decrease in absorbance resulted from non-specific loss of NADH (i.e., occurring in the absence of exogenous substrate). Treatment of sonified cells with 20 μg/ml of trypsin for 5 min resulted in loss of 60–80% of the malate dehydrogenase activity. Incubation of cell preparations at 35.5°, after sonification in culture medium, revealed the half-life of malate dehydrogenase activity to be greater than 15 hr.

Alkaline phosphatase activity was measured by the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate according to the method of Garen and Levinthal (18). Results from duplicates are expressed in μmol of *p*-nitrophenol formed/min per g wet weight of cells. Proteins were determined by the method of Lowry *et al.* (19). DNA content of the supernatant culture medium was determined by the Hinegardner modification (20) of the method of Kissane and Robins (21).

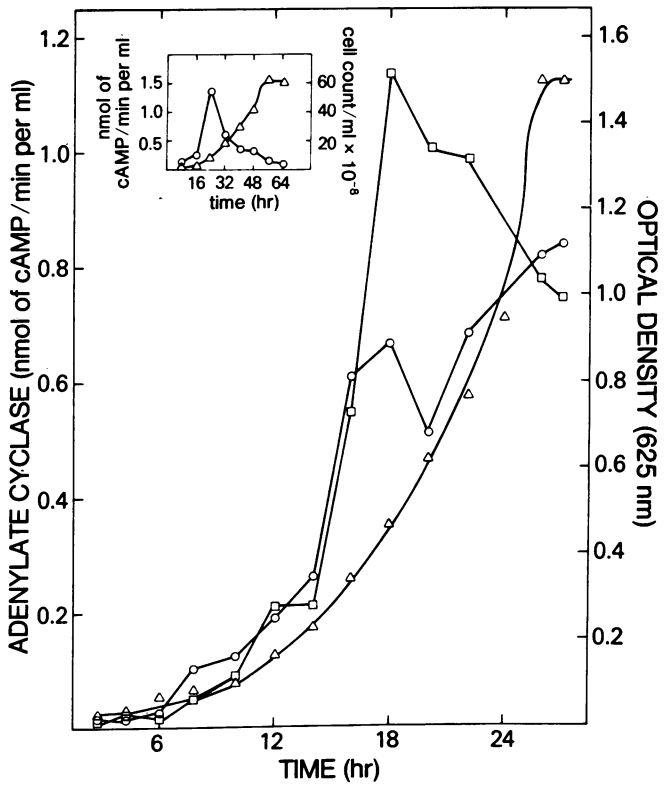

FIG. 1. Time course of *B. pertussis* adenylate cyclase expression by intact cells, and enzyme accumulation in the supernatant culture medium. Organisms were grown in modified Stain-Scholte medium and 7 ml aliquots were removed at 2 hr intervals. Optical density was measured at 625 nm and the cells were separated from the supernatant medium by centrifugation. Intact cells, resuspended in 7 ml of Tricine buffer (60 mM, pH 7.4), and supernatant medium were assayed for adenylate cyclase activity, which is expressed as nmol of cAMP formed per min/ml of either culture medium or cells. The inset shows the time course of supernatant enzyme activity from a slower growing culture. Intact cell activity, □—□; culture supernatant activity, ○—○; optical density at 625 nm △—△.

RESULTS

The supernatant medium of a 24 hr culture of exponentially growing *B. pertussis* cells contained abundant adenylate cyclase activity (300–900 pmol of cAMP/min per ml). This activity represents the soluble (centrifugation at 100,000 × *g* for one hr) enzyme which has been purified and characterized (1). During studies to determine the source of the supernatant enzyme, it was discovered that in addition to the activity found in the culture medium, a large amount of adenylate cyclase activity was associated with the organisms themselves. A significant proportion of this enzyme activity could be assayed using the intact organisms, which were able to form [^{32}P]cAMP from exogenous [α-^{32}P]ATP (200–1200 nmol of cAMP/min per g wet weight of cells).

The relationship between the growth of the bacteria, the adenylate cyclase activity in the supernatant medium, and that

Table 1. Distribution of *B. pertussis* enzymes*

	Malate dehydrogenase, μmol of NADH oxidized per min	Alkaline phosphatase, μmol of *p*-nitrophenol formed per min	Adenylate cyclase, nmol of cAMP formed per min
Culture supernatant	0	0	340 (18%)
Intact organisms	1.25 (1.7%)	34 (2.4%)	778 (40%)
Spheroplast lysate	75.3	1330	1580 (82%)
Total activity	75.3 (100%)	1330 (100%)	1920 (100%)

Organisms from a 20–24 hr culture were harvested as described and the intact organisms were resuspended in Tris-saline buffer (0.01 M, pH 7.6) to a concentration of 5 mg wet weight of cells per ml. An aliquot of supernatant culture medium and resuspended cells were removed for assay, and the remaining cells were disrupted by the lysozyme-EDTA osmotic shock procedure. The spheroplast lysate was assayed after DNase treatment. Total activity of each enzyme was taken to be the sum of the supernatant culture medium value and that of the spheroplast lysate. The supernatant adenylate cyclase value was corrected to that volume of medium containing 1 g of cells.

* All values are reported per g wet weight of cells.

Table 2. Effect of trypsin treatment and sonication on measurable *B. pertussis* adenylate cyclase and malate dehydrogenase*

	Adenylate cyclase, nmol of cAMP formed per min		Malate dehydrogenase, μmol of NADH oxidized per min	
	Control	Trypsin-treated	Control	Trypsin-treated
Whole cells	294	14	4.1	4.1
Cell sonicate	696	63	53.4	51.4

Organisms from a 20–24 hr culture were harvested by centrifugation, resuspended in fresh culture medium, and divided into control and trypsin groups. Two ml of cells (2.5–4.0 mg wet weight of cells) were treated with 20 μg/ml of trypsin for 5 min at 37° and the reaction was stopped with 20 μg/ml of lima bean trypsin inhibitor. The control and trypsin treated cells were then sonicated in 20 sec bursts, for a total of 160–200 sec, and assayed as described for adenylate cyclase and malate dehydrogenase activity.
* All values reported per g wet weight of cells.

expressed by intact organisms is shown in Fig. 1. In a culture reaching the stationary phase in 25 hr, the soluble supernatant enzyme activity increased rapidly during early exponential growth and more slowly at 18–24 hr. In contrast, a culture which began with a smaller inoculum and reached the stationary phase at 56 hr had a supernatant adenylate cyclase activity which peaked and began to decline at 24–30 hr. The reason for the eventual decline of supernatant medium and intact cell activities is not clear.

In order to localize the adenylate cyclase activity expressed by intact organisms, the distribution of malate dehydrogenase, alkaline phosphatase, and adenylate cyclase was compared in *B. pertussis* culture supernatant, intact cells, and spheroplast lysates (Table 1). The total activity of each enzyme was taken as the sum of the culture supernatant activity and that of the spheroplast lysate. Whereas a substantial portion of the total adenylate cyclase was found in the culture supernatant (18%) or was expressed by intact organisms (40%), malate dehydrogenase and, somewhat surprisingly, alkaline phosphatase appeared to be intracellular enzymes in this organism, manifesting only 1.7 and 2.4% of their total activities, respectively, in intact cells and none in the supernatant medium. Additional evidence against significant cell breakage was provided by the finding that no DNA could be detected in the cell-free culture medium. Disruption of the *B. pertussis* organisms by sonification yielded results essentially equivalent to those from spheroplast lysis. A small proportion of the total malate dehydrogenase and 40% of the adenylate cyclase were expressed by the whole cells (Table 2). Although the remaining 92–98% of the malate dehydrogenase activity was presumed to be intracellular, it was not clear whether the remaining 60% of the adenylate cyclase activity, liberated during cell disruption, was intracellular or extracytoplasmic but not measurable in intact cells.

If the bulk of *B. pertussis* adenylate cyclase was located outside the cytoplasmic membrane, then the enzyme should be susceptible to mild proteolysis which should have no effect on the intracellular enzyme, malate dehydrogenase. The treatment of fresh intact *B. pertussis* organisms with as little as 2 μg/ml of trypsin, for 5 min at 37°, destroyed 96% of the adenylate cyclase activity expressed by the intact cells (Table 2). Subsequent sonification of the trypsin-treated cells revealed their total remaining adenylate cyclase activity to be 63 nmole of cAMP/min per g wet weight of cells, or 9% of the activity found in sonified untreated cells. This residual fraction of adenylate cyclase activity remained constant when trypsin treatment was varied over a time span of 5–20 min with trypsin concentrations of 2–20 μg/ml. When the cells were sonified prior to trypsin treatment, greater than 99.5% of the adenylate cyclase activity was destroyed. These findings, thus, suggest that the residual 7–9% of adenylate cyclase activity represents a true intracellular compartment. In contrast, the total malate dehydrogenase activity released by sonication of trypsin-treated cells was the same as that from control cells, and indicates that the trypsin had no effect on this intracellular enzyme. This difference in response to trypsin treatment indicated that most of the adenylate cyclase was extracytoplasmic, rather than intracellular in location.

When the adenylate cyclase activity of *B. pertussis* culture supernatant and intact cells was compared to those of *E. coli* and *B. liquefaciens* (Table 3), the *B. pertussis* activities exceeded the maximum values of the other organisms by ten- to several thousandfold. In addition, the pretreatment of intact

Table 3. Maximum adenylate cyclase activity expressed by culture supernatant and intact cells of *E. coli*, *B. liquefaciens*, and *B. pertussis*

	Adenylate cyclase activity			
	Culture supernatant, pmol of cAMP formed/min per ml		Intact cells, pmol of cAMP formed/min per mg of protein	
	6 hr	24 hr	6 hr	24 hr
E. coli	2.4[a]	0.8	1.3[a]	5.1
B. liquefaciens	2.7	0.9	0.2[a]	2.5
B. pertussis[b]	28	674	3640	4770

At 6 and 24 hr, a sample of organisms was separated from the medium by centrifugation and the cells were resuspended in Tricine buffer (60 mM, pH 7.4) to their original concentration. Supernatant culture medium and resuspended cells were assayed for adenylate cyclase activity. The *B. liquefaciens* samples were assayed in the presence of 5 mM pyruvate. The values shown represent the maximum activities for *E. coli* and *B. liquefaciens* intact cells and culture medium at 6 and 24 hr. The media yielding these maximum values was modified Stainer-Scholte medium with 0.1% glucose unless noted otherwise.
[a] Bacto-Tryptic soy broth with 0.1% glucose.
[b] Modified Stainer-Scholte medium.

Table 4. Adenylate cyclase activity of intact control and toluene-treated *E. coli* and *B. pertussis*

	Adenylate cyclase activity, pmol of cAMP/min per mg of protein					
	E. coli			*B. pertussis*		
	Control	Treated	Treated/control	Control	Treated	Treated/control
Centrifuged	2.0	30.5	15.2	5260	2650	0.5
Filtered	0.4	12.5	31.2	4730	3055	0.64

E. coli and *B. pertussis* were cultured for 24 hr on Stainer-Scholte medium and harvested either by centrifugation (6000 × *g* for 30 min) or filtration (0.45 μm Millipore filter). Cells were resuspended in the same volume of 60 mM Tricine buffer containing 20 mM K_2HPO_4 (final pH 7.4). Samples were assayed for adenylate cyclase activity before and after 5 min treatment with 1% toluene.

organisms with 1% toluene before adenylate cyclase assay had opposite effects in *E. coli* and *B. pertussis* (Table 4). The measurable adenylate cyclase of intact *E. coli* was very low, but was increased 15- to 30-fold by toluene treatment. The adenylate cyclase activity of intact *B. pertussis*, on the other hand, was inhibited 36–50% by exposure to the toluene.

DISCUSSION

The data provided here show that *B. pertussis* adenylate cyclase fulfills two basic criteria for extracytoplasmic localization: (i) expression of the enzyme activity by the intact organism using an exogenous, nonpermeating substrate; and (ii) accumulation of the enzyme in the culture medium during exponential growth. In addition, we would suggest a third criterion, namely, sensitivity of the activity to brief treatment with a proteolytic enzyme, without damage to intracellular components. That the presence of adenylate cyclase in the culture medium is not due to leakage from broken cells is suggested by the following: (i) the absence of intracellular marker enzymes in the medium; (ii) the absence of DNA in the culture medium; and (iii) the accumulation of extracellular adenylate cyclase activity during exponential growth and its decline during the stationary phase of growth.

Our findings suggest the existence of four compartments of adenylate cyclase in *B. pertussis* (Fig. 2). The first compartment (A) consists of the enzyme in the supernatant culture medium which contributes up to 20% of the total adenylate cyclase present during mid-exponential growth. The remaining adenylate cyclase is associated with the cell and 91–93% of it is extracytoplasmic (destroyed by trypsin treatment of the intact cell, under conditions in which intracellular enzymes are unaffected). A portion of this cell-associated enzyme activity (20–45% of the total adenylate cyclase) can be measured with intact cells and an exogenous ATP concentration of 1 mM (compartment B). Compartment C consists of the remainder of the extracytoplasmic adenylate cyclase and comprises 40–60% of the total enzyme activity. It is possible to measure a small part of the C compartment enzyme without cell disruption, by increasing the exogenous ATP concentration to 20 mM. Hence, the dividing line between compartments B and C is a function of the exogenous ATP concentration. Nevertheless, compartment C contains a substantial portion of adenylate cyclase that is not measurable without sonification, but is extracytoplasmic by virtue of its sensitivity to trypsin. This is similar to the example of *E. coli* periplasmic alkaline phosphatase, in which the intact organisms are able to express as much as 93% of the total cell lysate activity only with high concentrations (10 mM) of some substrates (8). Finally, there appears to be a small (7–9% of the total adenylate cyclase) compartment (D) which is either intracellular or in some manner protected from destruction during trypsin treatment of the intact cell. This portion of adenylate cyclase is measurable and is susceptible to trypsin only after disruption of the cell.

As a control in the study of this unique extracytoplasmic adenylate cyclase, the intact cell and supernatant activities of the *B. pertussis* enzyme were compared to those of *E. coli* and *B. liquefaciens*. The *E. coli* adenylate cyclase is apparently membrane-bound (90% is found in the 150,000 × *g* pellet) and is inhibited by α-keto acids (4, 5). The *B. liquefaciens* enzyme, on the other hand, is soluble (105,000 × *g* for 1 hr) in cell lysates and is markedly stimulated by pyruvate and other α-keto acids (2, 22). Our data show that relative to *B. pertussis*, these other organisms have essentially no extracellular adenylate cyclase

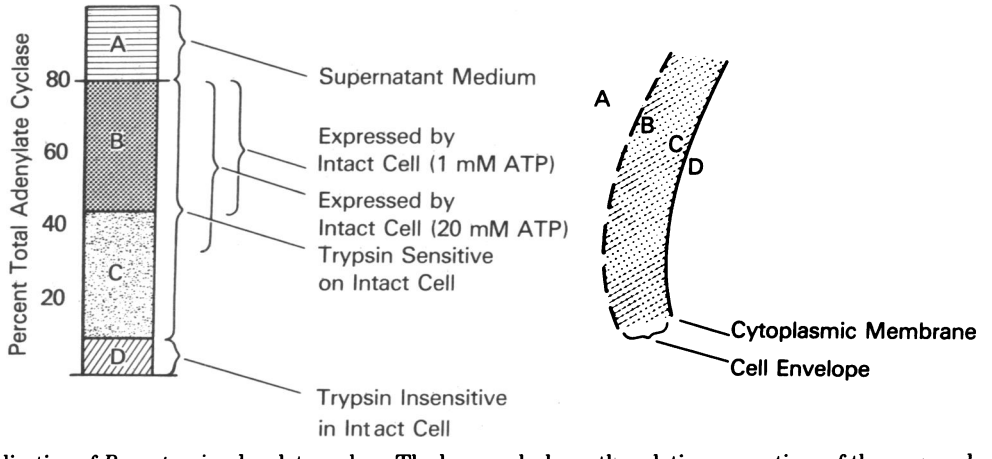

FIG. 2. The localization of *B. pertussis* adenylate cyclase. The bar graph shows the relative proportions of the proposed compartments and indicates the operational basis for each one. The diagram depicts one possible model for the relationship of each compartment to the cell envelope and cytoplasmic membrane.

activity. Indeed, the low activities can be accounted for by a small (1–5%) contamination by broken cells.

While numerous functions for bacterial intracellular adenylate cyclase have been described (23–26), the significance of a predominantly extracellular adenylate cyclase is unclear. Current studies do, however, indicate that this unusual adenylate cyclase may be associated with or contributing to known toxic effects of the organism (27, 28).

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